Subtype-Specific Genes that Characterize Subpopulations of Callosal Projection Neurons in Mouse Identify Molecularly Homologous Populations in Macaque Cortex

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Abstract

Callosal projection neurons (CPN) interconnect the neocortical hemispheres via the corpus callosum, and are implicated in associative integration of multi-modal information. CPN have undergone differential evolutionary elaboration, leading to increased diversity of cortical neurons – and more extensive and varied connections in neocortical gray and white matter – in primates compared to rodents. In mouse, distinct sets of genes are enriched in discrete subpopulations of CPN, indicating the molecular diversity of rodent CPN. Elements of rodent CPN functional and organizational diversity might thus be present in the further elaborated primate cortex.

We address the hypothesis that genes controlling mouse CPN subtype diversity might reflect molecular patterns shared among mammals that arose prior to the divergence of rodents and primates. We find that, while early expression of the examined CPN-enriched genes, and post-migratory expression of these CPN-enriched genes in deep layers are highly conserved (e.g. *Ptn, Nnmt, Cited2, Dkk3*), by contrast, the examined genes expressed by superficial layer CPN show more variable levels of conservation (e.g. *EphA3, Chn2*). These results suggest that there has been evolutionarily differential retraction and elaboration of superficial layer CPN subpopulations between mouse and macaque, with independent derivation of novel populations in primates. Together, these data inform future studies regarding CPN subpopulations that are unique to primates and rodents, and indicate putative evolutionary relationships.

Key Words (5 limit): rodent , corpus callosum, development, evolution, primate

Introduction

The neocortex is the seat of complex cognitive, perceptive, and motor function in mammals. As such, it has undergone dramatic expansion throughout mammalian evolution, with increased neocortical size and complexity in primates not found in rodents (Rakic P 2009). Because a fossil record of soft tissues, including the nervous system, is lacking (de Sousa AAW, B.A. 2007), the field must rely on the identification of conserved features across contemporary species to determine those that likely arose from common ancestors.

Since the divergence of mammalian ancestors from the sauropsid ancestors of reptiles and birds over 315 million years ago, the cortex has undergone considerable expansion, with a substantial, disproportionate increase in neuronal numbers in the superficial cortical layers II-IV among mammalian species (Reiner A 1991; Marín-Padilla M 1992; Reiner A 1993; Aboitiz F et al. 2003), with the primate cortex exhibiting greater expansion than rodents (Herculano-Houzel S 2012). In terms of neuron number, computational analyses estimate that mice have about 100 times fewer brain neurons than monkeys, and that monkeys, in turn, have about 10-15 times fewer brain neurons than humans, with a 15-fold increase in brain size from mouse to human using Jerison's encephalization quotient (Jerison HJ 1973; Braitenberg V 2001; Fish JL et al. 2008; Mota B and S Herculano-Houzel 2012). Investigation of the similarities and differences among these cortices continues to be an active area of research.

The corpus callosum (CC) and callosal projection neurons (CPN) are found exclusively in placental mammals (eutheria) (Aboitiz F and J Montiel 2003; Mihrshahi R 2006). CPN interconnect the two cortical hemispheres, and, with the exception of primate area VI(Dehay C et al. 1988; Chalupa LM et al. 1989), the expansion of superficial cortical layers correlates reasonably well with that of CPN, which are mostly located in superficial cortical layers (~90% in primates; ~80% in rodents) (Isseroff A et al. 1984; Meissirel C et al. 1991). Primate superficial layers comprise discrete layers II and III, containing multiple distinct sublayers that are easily distinguished at the histological level, while rodents and other small-brained mammals have less cytoarchitectural specification in these layers and rodent layer II/III is

histologically a single, indivisible layer (Lund JS 1973; Rakic P and DR Kornack 2001; Smart IH et al. 2002) (**Figure 1A, B**).

With delineation of layers II, IIIa, and IIIb in primate cerebral cortex, it is possible to address distinct cell types that populate these neuroarchitecturally distinct laminar units. Compared to rodents, primates exhibit expansion of cortical superficial layers, as well as expansion of cortical and interhemispheric white matter, raising the possibility that expansion of superficial layers might be, at least in part, due to increased CPN populations. However, while CPN in mice are evenly distributed throughout layer II/III (See **Figures 1A** and **6**), essentially all superficial layer primate CPN in sensorimotor cortex are restricted to layer III, and more specifically, are concentrated in deeper layer III (IIIb) (Jones EG and SP Wise 1977; Killackey HP et al. 1983; Killackey HP and LM Chalupa 1986; Manzoni T et al. 1986; Bullier J et al. 1990; Meissirel C et al. 1991). These data suggest either that mouse layers II/III contain a mixture of all of these neuron types that segregated following expansion in primate cortex, and/or that new neuron types have emerged to populate the expanded regions of primate cortex.

Although routine histology in mouse fails to reveal the cytoarchitectural diversity evident in primate cortex, recently published data on the molecular diversity of rodent CPN identify diverse neuronal subtypes that group into sublayers of the superficial cortical layers (Molyneaux BJ et al. 2009). These data suggest the possibility that similar neuronal diversity might exist in both rodents and primates. This hypothesis is further supported by the fact that, while in monkey, feedforward and feedback associative pathways are overwhelmingly segregated to layers IIIb, and IIIa, respectively, they are dispersed in a "salt-and-pepper" fashion in mouse superficial layers (Markov NT et al. 2014). Newly identified molecular controls over CPN subtype diversity in mice might reflect evolutionarily older, shared molecular expression and function, not revealed by routine histology. To investigate this hypothesis, we took advantage of the already existing knowledge of cell type-specific gene expression in *bona fide* mouse CPN, previously identified via retrograde labeling (Molyneaux, et al. 2009). We compared mouse expression patterns of representative examples of these same genes, with those of macaque at similar developmental stages.

The enlargement of the neocortex and its superficial layers follows the emergence and expansion of a specialized precursor pool: the subventricular zone (SVZ) (Smart IH et al. 2002; Lukaszewicz A et al. 2005; Kriegstein A et al. 2006). Basal progenitors of the SVZ transiently express Tbr2, which is a critical regulator of neurogenesis from the SVZ (Arnold SJ et al. 2008) and Tbr2 lineage tracing in mouse reveals that basal progenitors can contribute to all cortical layers. Cellular and molecular evidence supporting the hypothesis that the basal progenitors of the SVZ, $Cux2^{+}$ VZ/SVZ progenitors, and the progenitors of the outer SVZ, preferentially contribute to superficial layers has been provided by a number of studies in multiple species (Tarabykin V et al. 2001; Smart IH et al. 2002; Zimmer C et al. 2004; Noctor SC et al. 2004; Lukaszewicz A et al. 2005; Wu SX et al. 2005; Dehay C and H Kennedy 2007; Kowalczyk T et al. 2009; Fietz SA et al. 2010; Franco SJ et al. 2012; Reillo I and V Borrell 2012; Betizeau M et al. 2013; Gil-Sanz C et al. 2014; Vasistha NA et al. 2014; Arcila ML et al. 2014; Gil-Sanz C et al. 2015).

Unlike many genome-wide, layer-specific, microdissection-based, or regional-specific gene expression analyses in primate and mouse (Donoghue MJ and P Rakic 1999a, 1999b; Yoneshima H et al. 2006; Wang WZ et al. 2009; Johnson MB et al. 2009; Belgard TG et al. 2011; Ip BK et al. 2011; Bernard A et al. 2012; Sorensen SA et al. 2015), the present investigation follows the cell-type driven logic of previous studies (Hevner RF et al. 2003; Arlotta P et al. 2005; Molyneaux BJ et al. 2009) to address the intersection of laminar molecular diversity *within* a specific population of neocortical projection neurons− CPN− between mouse and primate during cortical development. Here, we report that many of the molecular controls acting to establish CPN diversity exhibit a conserved laminar expression between mouse and macaque during corticogenesis, but with some notable distinctions, predominantly with respect to the superficial layer CPN.

Materials and Methods

In situ hybridization candidate selection and probe design

Gene candidates for this study are expressed by purified bona fide CPN in developing mouse (validated by CPN subtype-specific neuronal FACS purification and Affymetrix microarray analysis in (Molyneaux BJ et al. 2009). Due to limited macaque tissue, we were not able to examine the entire set of genes reported in Molyneaux, et al.; rather we selected representative examples from each laminar division grouping (also considering robust expression in mouse).

All macaque *in situ* hybridization probes were designed to closely match reported homologous regions of corresponding mouse mRNA (Molyneaux BJ et al. 2009). Optimized primers were chosen in order to amplify between 400 and 900 bp of this conserved region (see supplementary Table S1). Since the macaque genome is not fully annotated, not all of the sequences could be obtained from verified sequences so some were obtained from NCBI predicted homologs or whole chromosome shotgun sequencing data. The *in situ* hybridization probes for E14 mouse were those designed by GenePaint.org, and were, therefore, not identical to those in Molyneaux, BJ, et al. 2009, nor are they necessarily homologous to the regions selected for the macaque probes developed specifically for this study.

RNA extraction, and first strand cDNA synthesis and library construction

Total RNA was extracted from frozen E85 macaque cortical tissue following the product insert instructions for organic RNA extraction with TRIzol ® Reagent (Invitrogen Life Sciences). First-strand cDNA was synthesized using Oligo(dT) primers (25ng/μL) and total RNA (0.5ng/μL), following the product insert instructions for Superscript II ® reverse transcriptase (Ambion Life Sciences). To remove RNA complementary to the cDNA, *E. coli* RNase H (0.1U/μL) was added and incubated at 37° C for 20 min. This single stranded DNA was then used as template for PCR (sequences of all primers used are listed in **supplementary Table S1**), and cloned into TOPO II ® (Invitrogen Life Sciences) bacterial vectors using appropriate restriction enzymes (New England Biolabs).

In situ hybridization

Nonradioactive colorimetric *in situ* hybridization was performed using probes labeled with dig-UTP. Sense probes were used as negative controls in all experiments. Each mouse age represents 3 individual brains with 2 technical replicates to control for variability. Due to limited tissue, E94 macaque data (roughly equivalent to E14 in mouse) represents a single individual brain with 2-3 technical replicates to control for variability, as noted in the figure legend. In addition, two developing macaque brains were analyzed at E108 (N=3 technical replicates) and E113 (N=2 technical replicates), a developmental stage roughly equivalent to P3 in mouse. Results were reported if expression patterns were consistent between repeats and across individuals within each stage, even if observed expression levels varied somewhat between individuals.

For *in situ* hybridization, 25 µm cryosections of fixed (4% PFA), cryoprotected (30% sucrose) E94 and E108/113 macaque forebrains were mounted on superfrost plus slides ® (Fisher Scientific) and postfixed in 4% PFA in PBS for 10 min., rinsed in PBS for 3 min., permeabilized in 0.3% Triton X-100 (Sigma) followed by RIPA cell lysis buffer [150 mM Sodium chloride, 1% Triton X-100, 1% deoxicholic acid sodium salt, 0.1% sodium dodesyl sulfate, 50 mM Tris-HCl, pH 7.5, 2mM EDTA], re-fixed in 4% PFA, acetylated for 15 min. in 0.1M triethanolamine/ 0.4% HCl/0.25% acetic anhydride (Sigma), and preybridizied for 1 hour in 65°C hybridization buffer [50% formamide, 5x SSC, 5x Denhardts [1µg/mL Ficoll 400, 1µg/mL Polyvinilpyrrolidone, 1µg/mL BSA] , 500µg/mL sheared salmon sperm DNA, 250µg/mL Yeast RNA]. Slides were incubated overnight (14-20 hours) at 65°C in 2µg/mL dig-labeled probe in hybridization buffer coverslipped with GeneFrame ® adhesive spacers (Thermo Scientific) in a well-humidified oven. Slides were then subjected to stringency washes in 2x SSC/ 50% formamide/ 0.1% Tween-20 at 65°C for 1 hour each. Sections were then rinsed in MABT [0.9M maleic acid (Sigma), 0.1M NaCl (Sigma), 0.0005% Tween 20 (Sigma), 0.175M NaOH (Sigma)] at RT, blocked in 10% goat serum in MABT, and incubated overnight in goat alkaline phosphatase-conjugated anti-dig (1:1000, Roche)

primary antibody in block, rinsed with MABT, followed by 30 a min. wash in alkaline phosphatase reaction buffer [100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20]. The alkaline phosphatase reaction was developed with NBT/BCIP in phosphatase reaction buffer, changing to a fresh solution every 1-4 hours at RT or every 6-9 hours at 4°C. When the reaction was judged complete (48-100 hours), tissue was rinsed in 0.1% Tween-20 in PBS, postfixed in 4% PFA for 30 min, counterstained for 1 min. in 1:10,000 4',6-diamidino-2-phenylindole (DAPI), and rinsed in 0.033M PB [27mM dibasic sodium phosphate, 6.3mM monobasic sodium phosphate]. Slides were coverslipped with Fluromount ® (Sigma), dried, and edges were protected with clear nail polish.

For E108/E113 tissue, an additional permeabilization step with proteinase K (Sigma) treatment [10µg /mL enzyme in 0.005M EDTA, 0.05M Tris, pH 8.0] for 10 min. at room temperature was added after the RIPA permeabilization.

All E14 mouse *in situ* hybridization images reported from GenePaint.org are from the C57/Bl6 strain, except for *Cited2*, which is only available from the NMRI strain.

Immunocytochemstry

Immunostaining was performed largely as described elsewhere (Molyneaux BJ et al. 2009). However, all immmunocytochemical reactions were performed on 25 µm cryosections of fixed (4% PFA), cryoprotected (30% sucrose) E94 or E108/113 macaque forebrain mounted on superfrost plus slides ® (Fisher Scientific). Sections were brought to room temperature, postfixed in 4% PFA in PBS (10 min), rinsed in PBS (3 min), and stained. Antigen retrieval in 0.1M citric acid (pH=6.0) for 10 min. at 95-98°C was used for CAV1 and LMO4 staining. Primary antibodies were used as follows: mouse-anti-CAV1, 1:500 (Cell Signaling #3238), goat-anti-LMO4, 1:200 (Santa Cruz Biotech SC- 11122), rabbit-anti-Nectin-3, 1:500 (Abcam ab63931).

Microscopy and image analysis

Images were acquired using a Nikon E90i microscope, equipped with a 1.5 megapixel cooled CCD digital camera (Andor Technology, Dublin, Northern Ireland), a 5 megapixel color CCD digital camera (Nikon Instruments, Melville, NY), and Elements acquisition software (Nikon Instruments, Melville, NY). All analysis was performed in primary somatosensory area (S1) unless otherwise noted. Images were processed using Adobe Photoshop.

Results

In situ hybridization protocol detects mRNA in macaque tissue

Because even subtle tissue preparation and worksite setup differences can influence the outcome of *in situ* hybridization results, and particularly because the primate tissue was prepared in one lab, and mouse tissue preparation and analysis of both primate and mouse were performed in the other, we chose a control probe (*Fezf2)* that is known to have a laminar expression in both mouse and primate, and that is highly conserved in layer V (high expression) and layer VI (low expression), (Molyneaux BJ et al. 2005; Ip BK et al. 2011; Kwan KY et al. 2008) Padmanabhan HK, Sohur US, and Macklis, JD, unpublished observations). In mouse, *Fezf2* is expressed at high levels by corticospinal motor neurons (CSMN) and related subcerebral projection neurons (SCPN), and at lower levels by corticothalamic projection neurons (and other related corticofugal neurons). *Fezf2* is necessary and sufficient (within cortical context) to produce CSMN/SCPN, but is excluded from CPN. We confirmed that the macaque tissue, handling, and protocols provided accurate *Fezf2* expression results.

In E94 macaque (late-corticogenesis), *Fezf2* is expressed in the cortical plate **(Figure 2A)**. At E108/113, when most neocortical projection neurons have largely completed their migration to their final laminar positions (Rakic P 1995), expression of *Fezf2* is high in layer V, and low in layer VI **(Figure 2B)**. This is similar to *Fezf2* expression in mouse layer V CSMN/SCPN (high level) and layer VI CThPN (low level) (Chen J-G et al. 2005; Arlotta P et al. 2005; Chen B et al. 2005; Molyneaux BJ et al. 2005; Rouaux C and P Arlotta 2010). These expression patterns in macaque confirm previous findings (Kwan KY et al. 2008; Ip BK et al. 2011), and validate the experimental strategy employed by the present study for comparing macaque and mouse gene expression.

Genes expressed by CPN early in mouse development are similarly expressed in macaque

Because some of the mouse CPN subtype-specific genes show high expression levels early in development, and because early gene expression is more likely conserved between species than late gene expression (Donoghue MJ and P Rakic 1999a, 1999b), we examined expression of four genes in E94

macaque cortex that are expressed in early mouse CPN development. Strikingly, the subset of genes examined that are expressed in the subventricular zone (SVZ) exhibit conserved expression in E14 mouse and E94 macaque, including the high expression of *Ptn* (**Figure 3A-B**). This is particularly pertinent with respect to CPN given the hypothesis of SVZ origin of superficial layer neurons (see Introduction). In addition, the post-mitotic, cortical plate gene expression of *Ptn* is also conserved, as is that of *Lmo4* and *Inhba* **(Figure 3A-F)**. Low-level *Inhba* expression extends into progenitors in macaque, but not in mouse. Additionally, *Cited2* expression, present in both the progenitor and post-mitotic regions, is also highly conserved between E14 mouse and E94 macaque (**Figure 4G-H**). Together, these data suggest that in both species' populations of progenitors and immature post-mitotic neurons are largely similar in terms of early gene expression at the ages examined.

The above data add to a growing body of work revealing similarities and differences between rodents and primates in progenitors and early post-mitotic neurons (Donoghue MJ and P Rakic 1999a, 1999b; Johnson MB et al. 2009; Ip BK et al. 2011; Bernard A et al. 2012). The present results, focused on laminar similarities between mouse and primate during corticogenesis, point to a general conservation of expression in the two species.

In the macaque E94 stage examined, only a fraction of CPN have terminated their migration, and the progenitors that produce the majority of the CPN population are still proliferating. In order to refine our investigation of the degree of gene expression conservation of CPN between primates and rodents, we investigated a later stage of corticogenesis (P3 in mouse and E108/113 in macaque). The similarities in laminar gene expression in both progenitor and post-mitotic zones **(Figure 3)** motivate further investigations (addressed below) into later similarities or divergences of gene expression.

Post migration CPN gene expression suggests homologous CPN populations in mouse and macaque

Multiple molecular subpopulations of CPN have been identified in mouse by examining unique sublaminar expression patterns of novel genes expressed by *bona fide* CPN during corticogenesis, after most neurons have migrated to their final laminar positions (Molyneaux BJ et al. 2009). Therefore, we

examined expression of four genes in E108/113 macaque cortex that are expressed in mouse CPN development at P3. Strikingly, the subset of genes examined that are expressed in both deep and superficial layers are largely conserved in laminar expression between mouse and macaque at these ages, including *Cited2*, *Chn2*, *EphA3*, and *Limch1* (**Figure 4A-L)**.

Cited2 is expressed in P3 mouse and E108/113 macaque in both superficial and deep layers (**Figure 4A-C).** *Chn2* is expressed in the deeper part of mouse layer II/III, and appears more highly expressed in layer III in macaque somatosensory cortex **(Figure 4D-F)**. Interestingly, *Chn2* is not expressed throughout macaque layer III at E108/113, but only in the more superficial part of this layer. This difference suggests that *Chn2*-expressing neurons in macaque at this age are more segregated than their P3 mouse counterparts. Even if they are fated to be overtaken by later-generated neurons, they comprise a more restricted sublaminar population in macaque than in mouse. If *Chn2*-expressing cells remain in the superficial portion of layer III and are not completely overtaken by later-born cells, they might include populations of local associative neurons, or even a subpopulation of dual CPN/intrahemispheric association neurons, since most CPN in macaque S1 are in deeper portions of layer III (Jones EG and SP Wise 1977; Killackey HP et al. 1983; Manzoni T et al. 1986; Meissirel C et al. 1991), though this would need to be verified by combining gene expression analysis with tract-tracing studies of connectivity.

EphA3^{+} neurons are restricted to the most superficial portion of layer II/III in mouse at P3, while they are more widely distributed throughout these layers in macaque at E108/113 (**Figure 4G-I,** (Donoghue MJ and P Rakic 1999b)). *Limch1* shows a complementary species-specific expression pattern, with its expression restricted to the most superficial cells of layer II in E108/113 macaque, but more broadly throughout layer II/III in P3 mouse (**Figure 4J-L**). These examples indicate that mouse CPN-enriched genes are also expressed in the cortical plate of the developing macaque at E108/113, where they show a laminar-specific pattern in agreement with the known distribution of CPN in primates, but with notable differences between mouse and macaque sub-laminar expression.

Differential developmental regulation of CPN genes between mouse and macaque

We observed that CPN-expressed genes display varying levels of conservation over developmental time. *Dkk3* exhibits strong conservation of early SVZ/CP expression and later layer VI cortical expression (**Figure 5A,B**). Analogously, *Nnmt* exhibits strong conservation of early SVZ/CP expression, and *Nnmt* is expressed later exclusively in the superficial portion of layer II, in both species. (**Figure 5A,B**). *Inhba* exhibits strong conservation of early SVZ/CP expression, but later is expressed in layer II in E108/113 macaque, while it is expressed more broadly throughout layer II/III in P3 mouse (**Figure 5C**).

Other genes, while present during development in both mouse and macaque cortex, do not show complete spatial or temporal conservation. A notable exception to the largely conserved early expression of developmentally regulated neocortical control genes is that of the cell adhesion and guidance molecule *Plexin-D1*. By E14 in mouse, *Plexin-D1* is strongly expressed by immature cortical plate neurons as they begin to extend their axons. However, no *Plexin-D1* expression is observed in E94 macaque cortical plate (**Figure 5D**). Post-migratory expression of *Plexin-D1* is conserved between mouse and macaque in layer V (**Figure 5D**). This lack of *Plexin-D1* expression in early macaque cortex does not appear to drive differential expression of *Plexin-D1* later in immature migrating neurons. Conversely, absence of *Gfra2* expression in early cortical plate is conserved between mouse and macaque. Interestingly, in addition to its conserved expression in layers Va and VI in P3 mouse and E108/113 macaque, *Gfra2* is also expressed at E108/113 by superficial layer neurons in macaque, suggesting a potentially novel neuronal subpopulation of superficial layer macaque CPN not present in mice **(Figure 5E)**.

A significant proportion of CPN (~10% in primates; ~20% in rodents) reside in deep layers and CPN are an appropriate deep layer population to study in order to better understand evolutionary diversity in deep layers of the cortex. Strikingly, all of the P3 deep layer mouse CPN genes examined (*Cited2* (**Figure 4A-C**), *Dkk3, Plexin-D1,* and *Gfra2* (**Figure 5B,D**)) are conserved in their deep layer expression in E108/113 macaque, suggesting that deep layer CPN could be evolutionarily relatively consistent between rodent and primate at these stages. Together, these data suggest that at E108/113, while expanded superficial layer CPN populations likely have refined and elaborated in primates, deep layer,

evolutionarily older CPN have remained largely consistent molecularly, and therefore potentially also functionally.

The selected subset of superficial layer CPN genes (*Chn2, Limch1, EphA3* (**Figure 4D-L**)*, Nnmt,* and *Inhba* (**Figure 5B, C**)) reveals populations of neurons that, while present in both rodent and macaque, are differentially elaborated. While the most superficial portions of layer II/III do not contain the dominant proportion of mouse CPN, extremely superficial layer II/III CPN subpopulations do exist in P3 mouse cortex (Molyneaux BJ et al. 2009); **Figure 6)**. Similarly, while layer II in macaque does not contain the dominant population of CPN at E108 (Killackey HP and LM Chalupa 1986; Dehay C et al. 1988; Bullier J et al. 1990; Meissirel C et al. 1991), there is a small CPN population present in somatosensory cortex layer II at E108 (Killackey HP and LM Chalupa 1986). While *Limch1* and other layer II CPN genes are likely not exclusive to CPN, and are probably also expressed by intrahemispheric association neurons, *Limch1* is expressed by CPN and uniquely in mouse superficial layer II/III at P3 (**Figure 6B**). Well-defined *Limch1* expression in macaque layer II (**Figure 4J-K**) might correspond to the small number of layer II CPN present in macaque at E108.

Subcellular and functional areal localization of CPN-expressed proteins suggest related functions for conserved genes expressed by CPN populations

The above analyses reveal both substantial molecular conservation and some divergence between populations of CPN in mouse and macaque in distinct laminar locations within somatosensory areas at two developmental times. While comparative genomics has revealed more changes in regulatory than in coding regions (Stern DL and V Orgogozo 2008), it remains unknown whether genes expressed by mouse and macaque CPN have shared functionality within these neurons. As a second assessment of potentially conserved gene function (after temporal- and laminar-specific expression, as described above), we investigated whether proteins developmentally expressed in macaque cortex share areal and/or subcellular localization with those expressed by mouse CPN.

LMO4 is differentially expressed in particular neocortical areas in mouse (Arlotta P et al. 2005; Sun T et al. 2005; Joshi PS et al. 2008; Huang Z et al. 2009; Cederquist GY et al. 2013). We investigated whether this is a shared feature between mouse and macaque, even though it might be modified by divergence in the complex process of cortical arealization between rodents and primates (Rakic P 1988; Donoghue MJ and P Rakic 1999a, 1999b). Strikingly, neocortical areal restriction of LMO4 is conserved (**Figure 7A, Figure S1A**). In E108 macaque, as in P3 mouse, LMO4 expression in motor cortex is throughout all neocortical layers, instead of the more restricted expression found in somatosensory cortex.

To compare subcellular localization of subtype-specific proteins, we selected three with distinct subcellular localization in mouse CPN: 1) Nectin-3, with axonal white matter localization in superficial layer CPN axons in the CC early in development (see (Molyneaux BJ et al. 2009); 2) CAV1, with cell body and membrane/neurite localization in a restricted subpopulation of CPN (Gaillard S et al. 2001; Boulware MI et al. 2007; Molyneaux BJ et al. 2009); and 3) LMO4, with nuclear localization in deep layer CPN of somatosensory cortex (Molnár Z and AF Cheung 2006; Huang Z et al. 2009; Cederquist GY et al. 2013). At E94, Nectin-3 is localized specifically in white matter tracts in developing macaque cortex **(Figure 7B, Figure S1B**). There is minimal localization in the cell-dense cortical plate, higher localization throughout the cell-sparse subplate, and very high, fiber-enriched localization in both the outer and inner fiber layers. Similarly, CAVI and LMO4 subcellular localizations are also highly conserved in both species. In E108 macaque, CAV1 is localized in neuronal cell membranes and neurites, as well as developing blood vessels (**Figure 7C, Figure S1C**) (Gaillard S et al. 2001; Sbaa E et al. 2006; Boulware MI et al. 2007; Molyneaux BJ et al. 2009), while LMO4 is localized in nuclei of deep layer somatosensory neocortical neurons **(Figure 7D, Figure S1C)**, as in P3 mouse (Arlotta P et al. 2005; Molnár Z and AF Cheung 2006; Huang Z et al. 2009; Cederquist GY et al. 2013). Together, these data suggest that gene product function could be shared by Nectin-3, CAV1, and LMO4 in early cortical development of rodents and primates, based on conserved protein subcellular localization and areal-specific expression between the mouse and macaque brains, motivating future functional analyses of these proteins in neocortical development. Conserved subcellular localization for these gene products within

shared neuronal populations adds further support to the hypothesis that not only did rodents and primates likely arise from shared ancestors with specific genetic controls over complex cortical neuronal populations already present, but also supports the hypothesis that gene products have similar functions. If further functional analyses validate conserved function, it would suggest applicability of rodent studies of these proteins' functions to understanding human development and disease.

Discussion

It is interesting to speculate that the presence of a molecularly diverse population of CPN in the superficial layers of the rodent cortex (Molyneaux BJ et al. 2009) is inherited from a common ancestor of rodents and primates that had already undergone early stages of the evolutionary expansion of these layers and conspicuously developed further complexity during subsequent evolution of the primate cortex. The present study begins to investigate this hypothesized evolutionary trajectory by analyzing comparative expression in primate of a representative selection of genes expressed by *bona fide* CPN in mouse. Genes that identify neurons located at distinct positions within mouse layer II/III support the hypothesis that specialization of distinct populations of CPN within the superficial layers are at least partially molecularly conserved in E14 and P3 rodent; and E94 and E108/113 primate cortex (**Table1, Figure S2)**, and possibly also with respect to connectivity and function. The work presented here directly investigates within the mouse and macaque cortex the expression of genes initially identified in distinct populations of mouse CPN, to provide insight into cortical evolution and elaboration of distinct populations of neocortical neurons. Enlargement of the superficial layers during cortical evolution is accompanied by the emergence and expansion of the SVZ (see Introduction), and the concomitant appearance of diverse progenitors with complex lineages largely fated to produce neurons of the superficial layers (Smart IH and GM McSherry 1982; Martinez-Cerdeno V et al. 2006; Molyneaux BJ et al. 2007; Woodworth MB et al. 2012; Custo-Grieg L et al. 2013; Betizeau M et al. 2013; Dehay C et al. 2015).

While rodents and primates both contain basally-located progenitors in the SVZ, the type, function, and specific localization of these progenitors differ significantly between mouse and macaque. The vast majority (~90%) of mouse intermediate SVZ progenitors are not polarized and are similar to the progenitors of the inner SVZ (ISVZ) in primate; they express *Tbr2*, but down-regulate *Pax6* (Fish JL et al. 2008; Fietz SA et al. 2010; LaMonica BE et al. 2012; Betizeau M et al. 2013). A distinct, polarized, radial basal progenitor type is highly expanded in primate OSVZ and these progenitors are more similar

to radial glial cells, both molecularly and morphologically (Smart IH et al. 2002; Lukaszewicz A et al. 2005; Fish JL et al. 2008; Fietz SA et al. 2010; Hansen DV et al. 2010; Betizeau M et al. 2013). Such progenitors constitute only a minute proportion of mouse progenitors, and these are not restricted to a specific zone. Polarization in these progenitors allows for more non-consuming, non-terminal divisions (Fish JL et al. 2008; LaMonica BE et al. 2012; Betizeau M et al. 2013). Importantly, these primate OSVZ progenitors exhibit stage-specific cell cycle dynamics, with a reduction of cell cycle duration in later stages of corticogenesis when neurons destined for the superficial layers are being generated. This suggests that the molecular and cellular properties of these progenitors contribute to increased superficial layer complexity and expansion in primates in comparison to rodents (Betizeau M et al. 2013; Arcila ML et al. 2014; Dehay C et al. 2015). Further, the radial glial-like progenitors of the OSVZ undergo both symmetric, self-renewing divisions, as well as asymmetric, neurogenic self-renewing divisions, and their cellular dynamics in primates vary at distinct developmental stages (Fietz SA et al. 2010; Hansen DV et al. 2010; Lui Jan H et al. 2011; Betizeau M et al. 2013). This capacity of OSVZ progenitors to undergo self-renewing asymmetric divisions in order to generate progenitors that can further proliferate greatly enhances neuronal output, and might have been an important evolutionary step in the expansion of the cortex, especially superficial layers (Dehay C et al. 2015).

We find that many of the early-expressed CPN genes in rodents and primates are highly consistent in laminar expression (e.g *Ptn, Inhba* (**Figure 3**), and *Dkk3, Nnmt*, (**Figure 5)**), but none are overtly restricted to the OSVZ, which reaches its maximum expansion in the primates. In addition, *Epha3,* expression in the cortical plate has been shown previously to have similar cortical plate expression in macaque earlier at E65, but restricted to caudal cortex (Donoghue MJ and P Rakic 1999a, 1999b). Taken together, conservation of these genes' expression might reflect a more ancient evolutionary origin of CPN than previously identified (at least during CPN generation), but also allows for the possibility of additional molecular complexity in primate progenitor domains than is observed in mouse (Arcila ML et al. 2014).

Because superficial neocortical layers contain the overwhelming majority of CPN, we hypothesized that some molecular controls over CPN development that segregate superficial layers in mice might reflect a common origin for CPN in the expanded superficial layers identified in primates. Some gene expression patterns identified in mouse at P3, including those of *Nnmt* and *Chn2,* very closely match those of E108/113 macaque. However, while mouse CPN molecular diversity suggests correlates to some more elaborated populations of primate CPN, the mouse repertoire does not appear to contain all of the primate molecular diversity, and has likely independently acquired unique populations critical for rodent cortical function. For example, while both *EphA3* and *Limch1* are expressed in rodent and macaque superficial layers, they are expressed by differentially expanded populations. This suggests that some CPN populations common to mouse and macaque were additionally elaborated in primates, whereas others were preferentially elaborated in rodents. These discrete populations might reflect functional processing differences that distinguish mouse and macaque, and might provide insight into CPN functions that are more dominant in human integrative function and cognition.

Compared to superficial layers, deep neocortical layers contain a high proportion of CPN, but have undergone less extensive expansion over mammalian evolution. Therefore, we hypothesized that most of the newly identified molecular controls over deep layer CPN development in mouse might likely be conserved in macaque. Indeed, all of the deep layer CPN gene expression patterns investigated are conserved between P3 mouse and E108/113 macaque (*Cited2, Dkk3, Plexin-D1,* and *Gfra2)*, supporting the hypothesis that deep layer CPN are likely relatively evolutionarily consistent between rodent and primate during corticogenesis. A more systematic comparative study would be needed to verify function, but these data suggest that, while the expanded superficial layer CPN populations have likely refined and elaborated in primates, deep layer, evolutionarily older CPN have remained largely consistent molecularly, and therefore potentially also functionally.

Unexpectedly, *Gfra2*, expressed exclusively in layer Va and VI CPN in P3 mouse, is, in addition to deep layer expression, also expressed strongly by superficial layer neurons in E108/113 macaque, suggesting a potentially entirely new population of CPN that acquired genetic controls first employed by

deep layer CPN. During development, deep layer CPN cross the midline prior to superficial layer CPN, and are related molecularly and by birthdate to corticofugal neurons, leading to the hypothesis that deep layer CPN were modified from existing corticofugal neuronal populations to become the first to cross the midline early in evolution (Lai T et al. 2008; Azim E et al. 2009; Fame RM et al. 2011). These results suggest the possibility that similar molecular pathways existing in basal populations could evolve for new and/or expanded functions in the *Gfra2*-expressing population of superficial layer neurons in E108/113 macaque. In this case, superficial layer CPN in E108/113 macaque might be derived from neurons forming very different connections, even from a subpopulation of corticofugal neurons. Data showing that genes (eg *Gfra2*) preferentially expressed in deep layers in the mouse exhibit an additional superficial layer expression in the macaque confirm and extend a large scale comparative study between mouse and human cortex (Zeng H et al. 2012), reporting a shift in gene expression from layer V in mouse to layer III in human cortex; hypothesized to contribute to differences in cortical function across species. Without functional data, divergence could also reflect passive deregulation of gene expression, while conservation argues strongly for conserved function. However for divergently expressed genes such as early *Plexin-D1*, which has been shown to be critical for neocortical development (Bribian A et al. 2014), passive deregulation is unlikely. Our current study further motivates functional analysis of the other divergent factors during corticogenesis.

In addition to CPN, both superficial and deep neocortical layers contain other populations of neurons. Deep layers predominantly contain corticofugal neurons. Superficial layers, also contain populations of non-callosal pyramidal neurons. In addition, all layers contain inhibitory interneurons, which could share a subset of gene expression with nearby excitatory neurons with common generation time and laminar/sub-laminar location. While the genes examined here in macaque were identified in a comparative microarray analysis in mice designed to identify genes more highly expressed by CPN than by CSMN and other subcerebral projection neurons in mice (also compared with corticothalamic projection neurons; Galazo, M.J. et al, unpublished), many are likely not exclusive to CPN. However, many of these genes are expressed quite specifically by mouse CPN compared to other cortical neuron

subtypes (Molyneaux BJ et al. 2009). In the macaque, we still lack an understanding of the connectivity of the neurons expressing these genes. It could be enlightening in the future to examine the gene expression of CPN identified by retrograde labeling during macaque corticogenesis, with the provision that the gene expression level is sufficient at developmental stages when CPN retrograde labeling is practical (around E120 to E130).

Complementary to comparative gene expression results in radially expanded neocortical neuronal populations, we also investigated the subcellular localization of CPN-expressed gene products in mouse and macaque to gain insight into potentially conserved gene function. We found that protein subcellular localization for Nectin-3, CAV1, and LMO4 is conserved between mouse and macaque, with Nectin-3 in axonal white matter, CAV1 in cell membranes and apical dendrites, and LMO4 in nuclei. Additionally, differential expression in distinct cortical areas is also conserved for LMO4. While conserved areal expression reflects conserved gene regulatory regions, identification of conserved protein localization additionally suggests conserved gene product function, which remains to be functionally investigated.

While differential segregation of gene expression has occurred in rodent and primate cortices, both contain CPN, and, therefore, it is likely that conserved gene expression at least partially maps onto conserved CPN populations. However, in some species of marsupials, in which distinct layers II and III are observed without CPN, evolutionary relationships between cortical neuron subtypes are less easily derived. Marsupials have fewer neurons compared to their rodent counterparts, but they do have a distinct SVZ with basal progenitors expressing conserved genetic factors, which appears later in cortical development, perhaps contributing to the smaller overall cortex (Cheung AF et al. 2010). Determining whether genes that identify CPN subpopulations in placental mammals are expressed in marsupial cortex might clarify how superficial layers without CPN developed from a common ancestor. Therefore, understanding relationships between gene expression in particular CPN subpopulations in mouse and macaque, and comparing with large cortices in marsupials, might provide evolutionary insight into the origins of marsupial connectivity, and cortical expansion; and generation of neuronal diversity across all mammalian species. The sauropsid cortex contains only three layers, which, share features with multiple

neuronal populations in mammals (Reiner A 1991; Suzuki IK et al. 2012; Belgard TG et al. 2013). Further investigation of subtype specific gene expression in those sauropsid cortical populations most closely related to CPN, in particular those genes with conserved expression in mouse and macaque, could reveal additional evolutionary relationship among these neuronal populations.

The data presented here provide the first comparison of the molecular identity of CPN subpopulations in rodent and primate. Such comparative information will likely be important for defining the extent to which rodent research can address the complex integrative functions of primate cortex. In the present study, the laminar distribution of recently identified mouse CPN genes in macaque at two developmental stages reveals the comparative homology of molecularly defined subpopulations that had not yet been previously described at histological, morphological, or anatomical levels. It is likely that distinct combinations of molecular developmental controls define key aspects of CPN diversity in both species – subtype-specific differentiation, axon collateralization, synaptic connectivity, and physiologic function – underlying their central roles in interhemispheric connectivity. Together, these data provide a foundation of evolutionary relationships that will inform future studies about those complex subpopulations of primate CPN that can be studied with molecular and genetic approaches in mouse, and those that are unique to primate.

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References

Aboitiz F, Montiel J. 2003. One hundred million years of interhemispheric communication: the history of the corpus callosum. Braz J Med Biol Res. 36: 409-420.

Aboitiz F, Morales D, Montiel J. 2003. The evolutionary origin of the mammalian isocortex: towards an integrated developmental and functional approach. Behav Brain Sci. 26: 535-552; discussion 552-585.

Arcila ML, Betizeau M, Cambronne XA, Guzman E, Doerflinger N, Bouhallier F, Zhou H, Wu B, Rani N, Bassett DS, Borello U, Huissoud C, Goodman RH, Dehay C, Kosik KS. 2014. Novel primate miRNAs coevolved with ancient target genes in germinal zone-specific expression patterns. Neuron. 81: 1255-1262.

Arlotta P, Molyneaux BJ, Chen J, Inoue J, Kominami R, Macklis JD. 2005. Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron. 45: 207-221.

Arnold SJ, Huang G-J, Cheung AFP, Era T, Nishikawa S-I, Bikoff EK, Molnár Z, Robertson EJ, Groszer M. 2008. The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone. Genes Dev. 22: 2479-2484.

Azim E, Jabaudon D, Fame RM, Macklis JD. 2009. SOX6 controls dorsal progenitor identity and interneuron diversity during neocortical development. Nat Neurosci. 12: 1238-1247.

Belgard TG, Marques AC, Oliver PL, Abaan HO, Sirey TM, Hoerder-Suabedissen A, Garcia-Moreno F, Molnar Z, Margulies EH, Ponting CP. 2011. A transcriptomic atlas of mouse neocortical layers. Neuron. 71: 605-616.

Belgard TG, Montiel JF, Wang WZ, Garcia-Moreno F, Margulies EH, Ponting CP, Molnar Z. 2013. Adult pallium transcriptomes surprise in not reflecting predicted homologies across diverse chicken and mouse pallial sectors. Proc Natl Acad Sci U S A. 110: 13150-13155.

Bernard A, Lubbers LS, Tanis KQ, Luo R, Podtelezhnikov AA, Finney EM, McWhorter MM, Serikawa K, Lemon T, Morgan R, Copeland C, Smith K, Cullen V, Davis-Turak J, Lee CK, Sunkin SM, Loboda AP, Levine DM, Stone DJ, Hawrylycz MJ, Roberts CJ, Jones

AR, Geschwind DH, Lein ES. 2012. Transcriptional architecture of the primate neocortex. Neuron. 73: 1083-1099.

Betizeau M, Cortay V, Patti D, Pfister S, Gautier E, Bellemin-Menard A, Afanassieff M, Huissoud C, Douglas RJ, Kennedy H, Dehay C. 2013. Precursor diversity and complexity of lineage relationships in the outer subventricular zone of the primate. Neuron. 80: 442-457.

Boulware MI, Kordasiewicz H, Mermelstein PG. 2007. Caveolin proteins are essential for distinct effects of membrane estrogen receptors in neurons. J Neurosci. 27: 9941-9950.

Braitenberg V. 2001. Brain size and number of neurons: an exercise in synthetic neuroanatomy. J Comput Neurosci. 10: 71-77.

Bribian A, Nocentini S, Llorens F, Gil V, Mire E, Reginensi D, Yoshida Y, Mann F, del Rio JA. 2014. Sema3E/PlexinD1 regulates the migration of hem-derived Cajal-Retzius cells in developing cerebral cortex. Nature communications. 5: 4265.

Bullier J, Dehay C, Dreher B. 1990. Bihemispheric Axonal Bifurcation of the Afferents to the Visual Cortical Areas during Postnatal Development in the Rat. The European journal of neuroscience. 2: 332-343.

Cederquist GY, Azim E, Shnider SJ, Padmanabhan H, Macklis JD. 2013. Lmo4 establishes rostral motor cortex projection neuron subtype diversity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 33: 6321-6332.

Chalupa LM, Killackey HP, Snider CJ, Lia B. 1989. Callosal projection neurons in area 17 of the fetal rhesus monkey. Brain Res Dev Brain Res. 46: 303-308.

Chen B, Schaevitz LR, McConnell SK. 2005. Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. Proc Natl Acad Sci USA. 102: 17184-17189.

Chen J-G, Rasin M-R, Kwan KY, Sestan N. 2005. Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex. Proc Natl Acad Sci USA. 102: 17792-17797.

Cheung AF, Kondo S, Abdel-Mannan O, Chodroff RA, Sirey TM, Bluy LE, Webber N, DeProto J, Karlen SJ, Krubitzer L, Stolp HB, Saunders NR, Molnar Z. 2010. The

subventricular zone is the developmental milestone of a 6-layered neocortex: comparisons in metatherian and eutherian mammals. Cerebral cortex. 20: 1071-1081.

Custo-Grieg L, Woodworth MB, Padmanabhan H, Galazo MJ, Macklis JD. 2013. Molecular logic of neocortical projection neuron specification, development, and diversity. Nat Rev Neurosci.

de Sousa AAW, B.A. 2007. The hominin fossil record and the emergence of the modern human central nervous system. In: T.M. Preuss JHK, ed. The Evolution of Primate Nervous Systems Oxford: Academic Press p 291-336.

Dehay C, Kennedy H. 2007. Cell-cycle control and cortical development. Nat Rev Neurosci. 8: 438-450.

Dehay C, Kennedy H, Bullier J, Berland M. 1988. Absence of interhemispheric connections of area 17 during development in the monkey. Nature. 331: 348-350.

Dehay C, Kennedy H, Kosik KS. 2015. The outer subventricular zone and primate-specific cortical complexification. Neuron. 85: 683-694.

Donoghue MJ, Rakic P. 1999a. Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. J Neurosci. 19: 5967-5979.

Donoghue MJ, Rakic P. 1999b. Molecular gradients and compartments in the embryonic primate cerebral cortex. Cereb Cortex. 9: 586-600.

Fame RM, MacDonald JL, Macklis JD. 2011. Development, specification, and diversity of callosal projection neurons. Trends Neurosci. 34: 41-50.

Fietz SA, Kelava I, Vogt J, Wilsch-Bräuninger M, Stenzel D, Fish JL, Corbeil D, Riehn A, Distler W, Nitsch R, Huttner WB. 2010. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat Neurosci. 13: 690-699.

Fish JL, Dehay C, Kennedy H, Huttner WB. 2008. Making bigger brains-the evolution of neural-progenitor-cell division. Journal of Cell Science. 121: 2783-2793.

Franco SJ, Gil-Sanz C, Martinez-Garay I, Espinosa A, Harkins-Perry SR, Ramos C, Muller U. 2012. Fate-restricted neural progenitors in the mammalian cerebral cortex. Science. 337: 746-749.

Gaillard S, Bartoli M, Castets F, Monaghan AP. 2001. Striatin, a calmodulin-dependent scaffolding protein, directly binds caveolin-1. FEBS Letters.

Gil-Sanz C, Espinosa A, Fregoso SP, Bluske KK, Cunningham CL, Martinez-Garay I, Zeng H, Franco SJ, Muller U. 2015. Lineage Tracing Using Cux2-Cre and Cux2-CreERT2 Mice. Neuron. 86: 1091-1099.

Gil-Sanz C, Landeira B, Ramos C, Costa MR, Muller U. 2014. Proliferative defects and formation of a double cortex in mice lacking Mltt4 and Cdh2 in the dorsal telencephalon. J Neurosci. 34: 10475-10487.

Hansen DV, Lui JH, Parker PRL, Kriegstein AR. 2010. Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature. 1-10.

Herculano-Houzel S. 2012. Neuronal scaling rules for primate brains: the primate advantage. Prog Brain Res. 195: 325-340.

Hevner RF, Daza RA, Rubenstein JL, Stunnenberg H, Olavarria JF, Englund C. 2003. Beyond laminar fate: toward a molecular classification of cortical projection/pyramidal neurons. Dev Neurosci. 25: 139-151.

Huang Z, Kawase-Koga Y, Zhang S, Visvader J, Toth M, Walsh CA, Sun T. 2009. Transcription factor Lmo4 defines the shape of functional areas in developing cortices and regulates sensorimotor control. Dev Biol. 327: 132-142.

Ip BK, Bayatti N, Howard NJ, Lindsay S, Clowry GJ. 2011. The corticofugal neuron-associated genes ROBO1, SRGAP1, and CTIP2 exhibit an anterior to posterior gradient of expression in early fetal human neocortex development. Cereb Cortex. 21: 1395-1407.

Isseroff A, Schwartz ML, Dekker JJ, Goldman-Rakic PS. 1984. Columnar organization of callosal and associational projections from rat frontal cortex. Brain Res. 293: 213-223.

Jerison HJ. 1973. Evolution of the brain and intelligence. New York,: Academic Press. xiv, 482 p. p.

Johnson MB, Kawasawa YI, Mason CE, Krsnik Z, Coppola G, Bogdanovic D, Geschwind DH, Mane SM, State MW, Sestan N. 2009. Functional and evolutionary insights into human brain development through global transcriptome analysis. Neuron. 62: 494-509.

Jones EG, Wise SP. 1977. Size, laminar and columnar distribution of efferent cells in the sensory-motor cortex of monkeys. J Comp Neurol. 175: 391-438.

Joshi PS, Molyneaux BJ, Feng L, Xie X, Macklis JD, Gan L. 2008. Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. Neuron. 60: 258-272.

Killackey HP, Chalupa LM. 1986. Ontogenetic change in the distribution of callosal projection neurons in the postcentral gyrus of the fetal rhesus monkey. The Journal of comparative neurology. 244: 331-348.

Killackey HP, Gould HJ, 3rd, Cusick CG, Pons TP, Kaas JH. 1983. The relation of corpus callosum connections to architectonic fields and body surface maps in sensorimotor cortex of new and old world monkeys. J Comp Neurol. 219: 384-419.

Kowalczyk T, Pontious A, Englund C, Daza RAM, Bedogni F, Hodge R, Attardo A, Bell C, Huttner WB, Hevner RF. 2009. Intermediate Neuronal Progenitors (Basal Progenitors) Produce Pyramidal-Projection Neurons for All Layers of Cerebral Cortex. Cerebral Cortex. 19: 2439-2450.

Kriegstein A, Noctor S, Martinez-Cerdeno V. 2006. Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nat Rev Neurosci. 7: 883-890.

Kwan KY, Lam MMS, Krsnik Z, Kawasawa YI, Lefebvre V, Sestan N. 2008. SOX5 postmitotically regulates migration, postmigratory differentiation, and projections of subplate and deep-layer neocortical neurons. Proc Natl Acad Sci USA. 105: 16021-16026.

Lai T, Jabaudon D, Molyneaux BJ, Azim E, Arlotta P, Menezes JR, Macklis JD. 2008. SOX5 Controls the Sequential Generation of Distinct Corticofugal Neuron Subtypes. Neuron. 57: 232-247.

LaMonica BE, Lui JH, Wang X, Kriegstein AR. 2012. OSVZ progenitors in the human cortex: an updated perspective on neurodevelopmental disease. Current Opinion in Neurobiology. 22: 747-753.

Lui Jan H, Hansen David V, Kriegstein Arnold R. 2011. Development and Evolution of the Human Neocortex. Cell. 146: 18-36.

Lukaszewicz A, Savatier P, Cortay V, Giroud P, Huissoud C, Berland M, Kennedy H, Dehay C. 2005. G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. Neuron. 47: 353-364.

Lund JS. 1973. Organization of neurons in the visual cortex, area 17, of the monkey (Macaca mulatta). The Journal of comparative neurology. 147: 455-496.

Manzoni T, Conti F, Fabri M. 1986. Callosal projections from area SII to SI in monkeys: anatomical organization and comparison with association projections. J Comp Neurol. 252: 245-263.

Marín-Padilla M. 1992. Ontogenesis of the pyramidal cell of the mammalian neocortex and developmental cytoarchitectonics: a unifying theory. J Comp Neurol. 321: 223-240.

Markov NT, Vezoli J, Chameau P, Falchier A, Quilodran R, Huissoud C, Lamy C, Misery P, Giroud P, Ullman S, Barone P, Dehay C, Knoblauch K, Kennedy H. 2014. Anatomy of hierarchy: feedforward and feedback pathways in macaque visual cortex. J Comp Neurol. 522: 225-259.

Martinez-Cerdeno V, Noctor SC, Kriegstein AR. 2006. The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. Cereb Cortex. 16 Suppl 1: i152-161.

Meissirel C, Dehay C, Berland M, Kennedy H. 1991. Segregation of callosal and association pathways during development in the visual cortex of the primate. The Journal of neuroscience : the official journal of the Society for Neuroscience. 11: 3297-3316.

Mihrshahi R. 2006. The corpus callosum as an evolutionary innovation. J Exp Zool B Mol Dev Evol. 306: 8-17.

Molnár Z, Cheung AF. 2006. Towards the classification of subpopulations of layer V pyramidal projection neurons. Neurosci Res. 55: 105-115.

Molyneaux BJ, Arlotta P, Fame RM, MacDonald JL, MacQuarrie KL, Macklis JD. 2009. Novel subtype-specific genes identify distinct subpopulations of callosal projection neurons. J Neurosci. 29: 12343-12354.

Molyneaux BJ, Arlotta P, Hirata T, Hibi M, Macklis JD. 2005. Fezl is required for the birth and specification of corticospinal motor neurons. Neuron. 47: 817-831.

Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD. 2007. Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci. 8: 427-437.

Mota B, Herculano-Houzel S. 2012. How the cortex gets its folds: an inside-out, connectivity-driven model for the scaling of Mammalian cortical folding. Front Neuroanat. 6: 3.

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nature neuroscience. 7: 136-144.

Rakic P. 1988. Specification of cerebral cortical areas. Science. 241: 170-176.

Rakic P. 1995. Radial versus tangential migration of neuronal clones in the developing cerebral cortex. Proc Natl Acad Sci U S A. 92: 11323-11327.

Rakic P. 2009. Evolution of the neocortex: a perspective from developmental biology. Nat Rev Neurosci. 10: 724-735.

Rakic P, Kornack DR. 2001. Neocortical expansion and elaboration during primate evolution: a view from neuroembryology. In: Falk D, Gibson KR, eds. In: Evolutionary anatomy of the primate cerebral cortex Cambridge, UK: Cambridge UP p 30-56.

Reillo I, Borrell V. 2012. Germinal zones in the developing cerebral cortex of ferret: ontogeny, cell cycle kinetics, and diversity of progenitors. Cereb Cortex. 22: 2039-2054.

Reiner A. 1991. A comparison of neurotransmitter-specific and neuropeptide-specific neuronal cell types present in the dorsal cortex in turtles with those present in the isocortex in mammals: implications for the evolution of isocortex. Brain Behav Evol. 38: 53-91.

Reiner A. 1993. Neurotransmitter organization and connections of turtle cortex: implications for the evolution of mammalian isocortex. Comp Biochem Physiol Comp Physiol. 104: 735-748.

Rouaux C, Arlotta P. 2010. Fezf2 directs the differentiation of corticofugal neurons from striatal progenitors in vivo. Nat Neurosci. 13: 1345-1347.

Sbaa E, Dewever J, Martinive P, Bouzin C, Frérart F, Balligand J-L, Dessy C, Feron O. 2006. Caveolin plays a central role in endothelial progenitor cell mobilization and homing in SDF-1-driven postischemic vasculogenesis. Circ Res. 98: 1219-1227.

Smart IH, Dehay C, Giroud P, Berland M, Kennedy H. 2002. Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. Cereb Cortex. 12: 37-53.

Smart IH, McSherry GM. 1982. Growth patterns in the lateral wall of the mouse telencephalon. II. Histological changes during and subsequent to the period of isocortical neuron production. J Anat. 134 (Pt 3): 415-442.

Sorensen SA, Bernard A, Menon V, Royall JJ, Glattfelder KJ, Desta T, Hirokawa K, Mortrud M, Miller JA, Zeng H, Hohmann JG, Jones AR, Lein ES. 2015. Correlated gene expression and target specificity demonstrate excitatory projection neuron diversity. Cerebral cortex. 25: 433-449.

Stern DL, Orgogozo V. 2008. The loci of evolution: how predictable is genetic evolution? Evolution. 62: 2155-2177.

Sun T, Patoine C, Abu-Khalil A, Visvader J, Sum E, Cherry TJ, Orkin SH, Geschwind DH, Walsh CA. 2005. Early asymmetry of gene transcription in embryonic human left and right cerebral cortex. Science. 308: 1794-1798.

Suzuki IK, Kawasaki T, Gojobori T, Hirata T. 2012. The temporal sequence of the mammalian neocortical neurogenetic program drives mediolateral pattern in the chick pallium. Dev Cell. 22: 863-870.

Tarabykin V, Stoykova A, Usman N, Gruss P. 2001. Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. Development. 128: 1983-1993.

Vasistha NA, Garcia-Moreno F, Arora S, Cheung AF, Arnold SJ, Robertson EJ, Molnar Z. 2014. Cortical and Clonal Contribution of Tbr2 Expressing Progenitors in the Developing Mouse Brain. Cereb Cortex.

Wang WZ, Oeschger FM, Lee S, Molnar Z. 2009. High quality RNA from multiple brain regions simultaneously acquired by laser capture microdissection. BMC Mol Biol. 10: 69.

Woodworth MB, Custo Greig L, Kriegstein AR, Macklis JD. 2012. SnapShot: cortical development. Cell. 151: 918-918 e911.

Wu SX, Goebbels S, Nakamura K, Nakamura K, Kometani K, Minato N, Kaneko T, Nave KA, Tamamaki N. 2005. Pyramidal neurons of upper cortical layers generated by NEX-positive progenitor cells in the subventricular zone. Proc Natl Acad Sci U S A. 102: 17172-17177.

Yoneshima H, Yamasaki S, Voelker CC, Molnar Z, Christophe E, Audinat E, Takemoto M, Nishiwaki M, Tsuji S, Fujita I, Yamamoto N. 2006. Er81 is expressed in a subpopulation of layer 5 neurons in rodent and primate neocortices. Neuroscience. 137: 401-412.

Zeng H, Shen EH, Hohmann JG, Oh SW, Bernard A, Royall JJ, Glattfelder KJ, Sunkin SM, Morris JA, Guillozet-Bongaarts AL, Smith KA, Ebbert AJ, Swanson B, Kuan L, Page DT, Overly CC, Lein ES, Hawrylycz MJ, Hof PR, Hyde TM, Kleinman JE, Jones AR. 2012. Large-scale cellular-resolution gene profiling in human neocortex reveals species-specific molecular signatures. Cell. 149: 483-496.

Zimmer C, Tiveron MC, Bodmer R, Cremer H. 2004. Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. Cerebral Cortex. 14: 1408-1420.

Captions to figures

Figure 1: Schematic representation and cytoarchitectural view of mouse and macaque somatosensory cortex.

(A) Schematic representation of developing and adult mouse and macaque cortex in S1, drawn to a common internal scale. **(B)** DAPI nuclear stain at mid-corticogenesis (E14/ E94) and after neuronal migration (P3/ E108) in macaque to show cytoarchitectural layers in S1.

Scale bars: **B**,100 μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; SP, subplate; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; CP, cortical plate; S1, primary somatosensory area; roman numerals indicate cortical layers. **A,** adapted from (Fame RM et al. 2011).

Figure 2: *Fezf2* is detectable in macaque tissue using current *in situ* hybridization approach.

(A) DAPI nuclear stain in E94 macaque for reference, followed by *in situ* hybridization for *Fezf2*at E94 in macaque and E14 in mouse, showing early similarities in expression between mouse and macaque. **(B)** DAPI nuclear stain in E108 macaque for reference, followed by *in situ* hybridization for *Fezf2* at E108 in macaque and P3 in mouse, confirming early similarities in expression between mouse and macaque, and validating the approach employed in these analyses. N=3 individual mice, N=2 technical replicates each. N=1 macaque, N=2 technical replicates.

Scale bars: **A**, **B**,100 μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; S1, primary somatosensory area; Roman numerals indicate cortical layers. E14 mouse *in situ* from GenePaint digital expression atlas www.genepaint.org.

Figure 3: At mid-corticogenesis (E14 and E94), early expressed CPN genes are largely similarly expressed in mouse and macaque

(A, B, C, D, E, F, G, H) *in situ* hybridization at E94 in macaque **(A, A' C, C', E, E', G, G')** and E14 in mouse **(B, B', D, D', F, F', H, H')** for early expressed CPN genes (*Ptn, Lmo4, Inhba, Cited2*) shows early similarities in expression between mouse and macaque. Low-level *Inhba* expression extends into progenitors in macaque, but not in mouse. N=1 macaque, N=3 technical replicates.

Scale bar: 100 μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; S1, primary somatosensory area; Roman numerals indicate cortical layers. E14 mouse *in situ* from GenePaint digital expression atlas www.genepaint.org.

Figure 4: At P3 in mouse, and at E108 and E113 in macaque, CPN genes reveal related populations in mouse and macaque superficial (*Cited2, Chn2, Epha3, Limch1*) and deep (*Cited2*) cortical layers. **(A-L)** *in situ* hybridization at E108in macaque **(A, D, G, J)**, at E113 in macaque **(B, E, H, K),** and at P3 in mouse (**C, F, I, L**), for *Cited2* in layers II/III and V; and **(A-C)**, *Chn2* **(D-F)**, *Epha3* **(G-I)**, and *Limch1* in superficial layers **(J-L)** showing molecular similarities between cellular populations in mouse and macaque cortex. **(A'- L')** detailed insets. N=3 individual mice, N=2 technical replicates each. N=1 E108 macaque, N=3 technical replicates. N= 1 E113 macaque, N=2 technical replicates.

Scale bars: **A- L,** 100 μm ; **A', B', D', E', G', G', H', J'. K',** 200 μm macaque; **C', F', I', L',**100 μm mouse. E, embryonic day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate.

Figure 5: Some CPN genes have largely analogous developmental expression patterns in mouse and macaque, but with some divergence.

(A) DAPI nuclear stain in E94 and E108 macaque for reference, horizontal bars indicate the extent of each named layer and are used to indicate dominant expression patterns in the following panels. **(B)** *in situ* hybridization at E94 in macaque and E14 in mouse for CPN genes with strong conservation of expression early in SVZ and CPN, as well as later at E108 and E113 (*Dkk3, Nnmt)* ***(C)*** *in situ* hybridization at E94 in macaque and E14 in mouse for *Inhba* shows early similarities in expression between mouse and macaque and more restricted expression later at E108 and E113. **(D)** *in situ* hybridization at E94 in macaque and E14 in mouse for *Plxnd1* reveal divergence in early expression, but conservation later at E108 and E113 in macaque and P3 in mouse. **(E)** *in situ* hybridization for *Gfra2* reveals that while early expression is conserved, at later times gene expression in deep layers is conserved with additional expanded gene expression into layer II. N=3 individual mice, N=2 technical replicates each. N=1 E108 macaque, N=3 technical replicates. N= 1 E113 macaque, N=2 technical replicates.

Scale bars: 200 μm macaque; 100 μm mouse. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; SP, subplate; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; CP, cortical plate; S1, primary somatosensory area; roman numerals indicate cortical layers. Horizontal gray bars indicate layers of dominant gene expression.

Figure 6: While superficial layer II/III does not contain the dominant proportion of CPN in mouse, superficial layer II/III CPN subpopulations do exist in P3 mouse cortex and express superficial layer II/III CPN genes, including *Limch1*.

(A) Retrograde labeling in mouse using CTB reveals CPN cell bodies in cortical layer II/III at P8, including superficial portions of layer II/III. **(B)** *in situ hybridization* combined with retrograde labeling using CTB reveals CPN cell bodies in superficial portions of layer II/III whose localization overlaps with expression of the CPN gene *Limch1* in mouse, suggesting that the layer II expression of *Limch1* in Macaque (Figure 4) might include CPN within that layer. **(B')** Detailed medial inset from **(B)**. **(B")** Detailed lateral inset from **(B)**.

Scale bars: **B**, 500 μm; **B', B"**, 100 μm. CPN, callosal projection neurons; CTB, B subunit of cholera toxin, P, postnatal day.

Figure 7: Functional areal and subcellular localization of CPN-expressed proteins suggests related functions in rodents and primates for conserved genes expressed by CPN populations.

(A, A', A") Areal restriction of LMO4 is conserved between mouse and macaque. LMO4 expression in motor cortex extends throughout all neocortical layers, and more restricted expression limited to deep layers is observed in somatosensory cortex.

(B, B', C, C', D. D') Three proteins with distinct subcellular localization in mouse CPN were selected for study of subcellular localization in macaque: 1) Nectin-3, with axonal white matter localization by superficial layer CPN axons in the CC; 2) CAV1, with cell body and neurite localization by a subpopulation of deep layer CPN; and 3) LMO4, with nuclear localization in deep layer CPN of somatosensory cortex. **(B, B')** At E94, Nectin-3 is localized specifically in white matter tracts in developing macaque cortex. There is low level localization in the cell-dense cortical plate, higher level localization throughout the cell-sparse subplate, and very high level, fiber-localization in both the outer and inner fibrous layers. **(C, C')** CAV1 is localized to neuronal cell membranes and neurites, as well as to developing blood vessels, as is seen in mouse ((Gaillard S et al. 2001; Boulware MI et al. 2007; Molyneaux BJ et al. 2009), and **(D, D')** LMO4 shows nuclear localization in deep layer somatosensory neocortical neurons, as has been shown in mouse. Empty arrowheads indicate membranous localization, and filled arrows indicate nuclear localization.

Scale bars: **A**, 1 mm; and **A', A"**, 100 μm; **B, C, D**, 100 μm; **C', D'**, 50 μm. E, embryonic day; P, postnatal day; ICS, incipient central sulcus; PCG, postcentral gyrus; S1, primary somatosensory area; M1, primary motor area; VZ, ventricular zone; SVZ, subventricular zone; SP, subplate; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; CP, cortical plate; roman numerals indicate cortical layers.

Table 1: Summary of laminar localization of CPN-enriched genes in Mouse and Macaque

Early (**A:** E94 Macaque; E14 Mouse) and late (**B:** E108/113 Macaque; P3 Mouse) laminar localization of gene expression examined in this study. Black-colored gene names show similar expression at these times between the two species. Red-colored gene names show differential expression at these times between the two species.

E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; S1, primary somatosensory area; Roman numerals indicate cortical layers.

A	E94 (Macaque)	E14 (Mouse)
CP	*Ptn, Lmo4, Inhba, Dkk3, Nnmt*	*Ptn, Lmo4, Inhba, Dkk3, Nnmt, Plxnd1*
SP		
SVZ	*Ptn, Dkk3, Nnmt, Inhba*	*Ptn, Dkk3, Nnmt*
VZ		

B	E108/113 (Macaque)	P3 (Mouse)
II (II/III sup)	*Limch1, Nnmt, Epha3, Inhba, Gfra2, Chn2*	*Limch1, Nnmt, Epha3, Inhba*
III (II/III deep)	*Cited2, Epha3*	*Cited2, Inhba, Chn2*
IV		
V	*Cited2, Dkk3, Plxnd1, Gfra2*	*Cited2, Dkk3, Plxnd1, Gfra2*
IV		

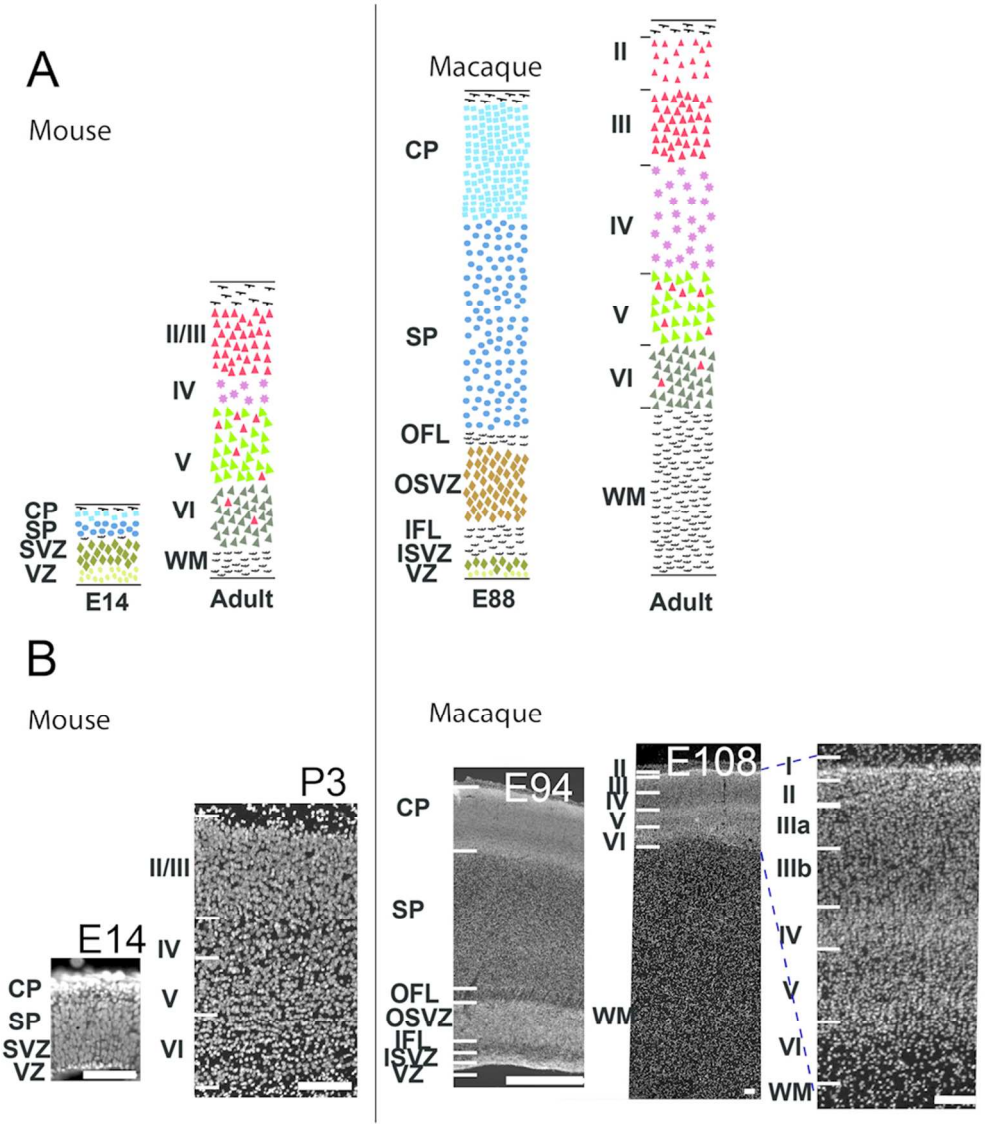

Figure 1: Schematic representation and cytoarchitectural view of mouse and macaque somatosensory cortex.

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Scale bars: B,100 μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; SP, subplate; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; CP, cortical plate; S1, primary somatosensory area; roman numerals indicate cortical layers. A, adapted from (Fame RM et al. 2011).

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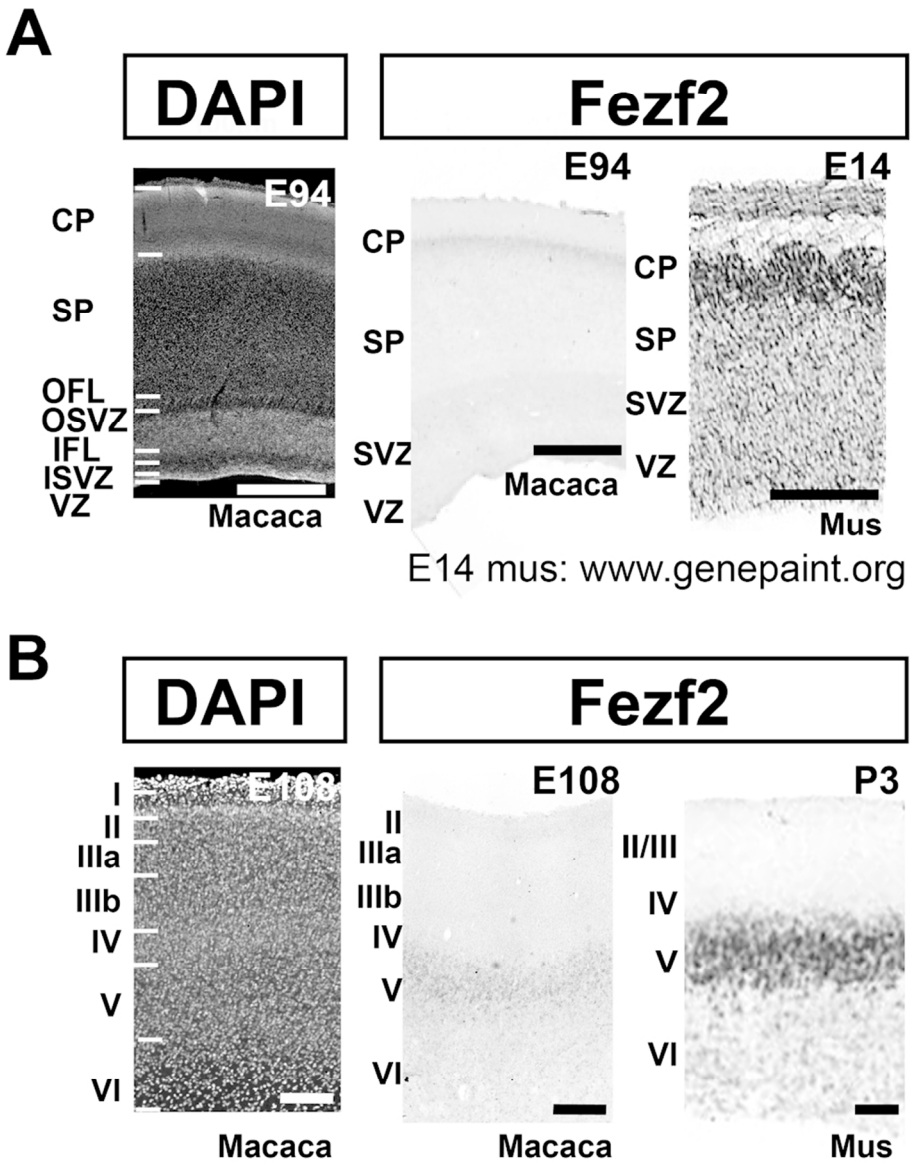

Figure 2: Fezf2 is detectable in macaque tissue using current in situ hybridization approach. (A) DAPI nuclear stain in E94 macaque for reference, followed by in situ hybridization for Fezf2at E94 in macaque and E14 in mouse, showing early similarities in expression between mouse and macaque. (B) DAPI nuclear stain in E108 macaque for reference, followed by in situ hybridization for Fezf2 at E108 in macaque and P3 in mouse, confirming early similarities in expression between mouse and macaque, and validating the approach employed in these analyses. N=3 individual mice, N=2 technical replicates each. N=1 macaque, N=2 technical replicates.

Scale bars: A, B,100 μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; S1, primary somatosensory area; Roman numerals indicate cortical layers. E14 mouse in situ from GenePaint digital expression atlas www.genepaint.org.

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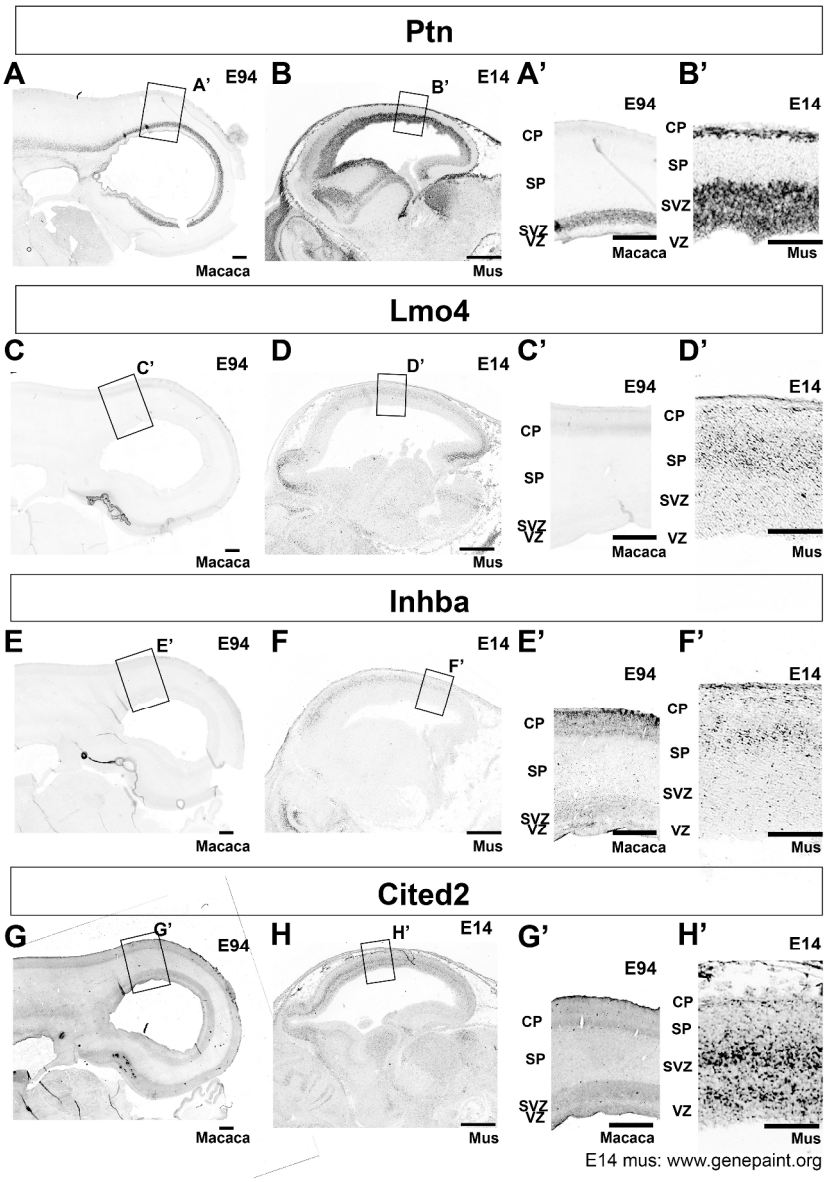

Figure 3: At mid-corticogenesis (E14 and E94), early expressed CPN genes are largely similarly expressed in mouse and macaque\r\n(A, B, C, D, E, F, G, H) in situ hybridization at E94 in macaque (A, A' C, C', E, E', G, G') and E14 in mouse (B, B', D, D', F, F', H, H') for early expressed CPN genes (Ptn, Lmo4, Inhba, Cited2) shows early similarities in expression between mouse and macaque. Low-level Inhba expression extends into progenitors in macaque, but not in mouse. N=1 macaque, N=3 technical replicates.\r\n\r\n\r\nScale bar: 100μm. E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; S1, primary somatosensory area; Roman numerals indicate cortical layers. E14 mouse in situ from GenePaint digital expression atlas www.genepaint.org.
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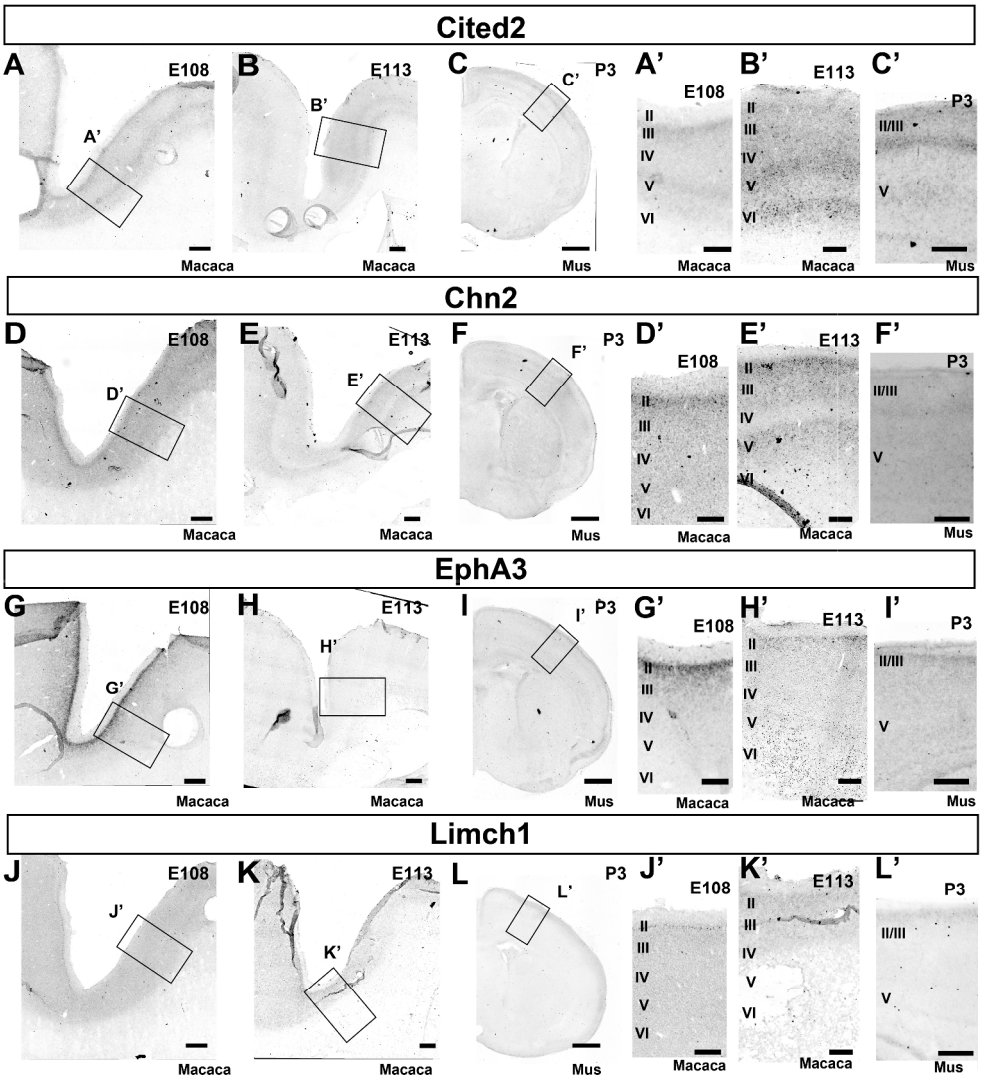

Figure 4: At P3 in mouse, and at E108 and E113 in macaque, CPN genes reveal related populations in mouse and macaque superficial (Cited2, Chn2, Epha3, Limch1) and deep (Cited2) cortical layers.\r\n(A-L) in situ hybridization at E108in macaque (A, D, G, J), at E113 in macaque (B, E, H, K), and at P3 in mouse (C, F, I, L), for Cited2 in layers II/III and V; and (A-C), Chn2 (D-F), Epha3 (G-I), and Limch1 in superficial layers (J-L) showing molecular similarities between cellular populations in mouse and macaque cortex. (A'- L') detailed insets. N=3 individual mice, N=2 technical replicates each. N=1 E108 macaque, N=3 technical replicates. N= 1 E113 macaque, N=2 technical replicates. \r\n\r\n\r\nScale bars: A- L, 100 µm ; A', B', D', E', G', H', J'. K', 200 µm macaque; C', F', I', L',100 µm mouse. E, embryonic day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate.
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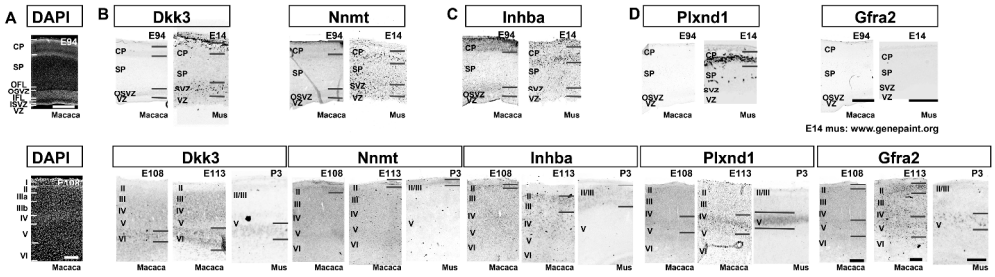

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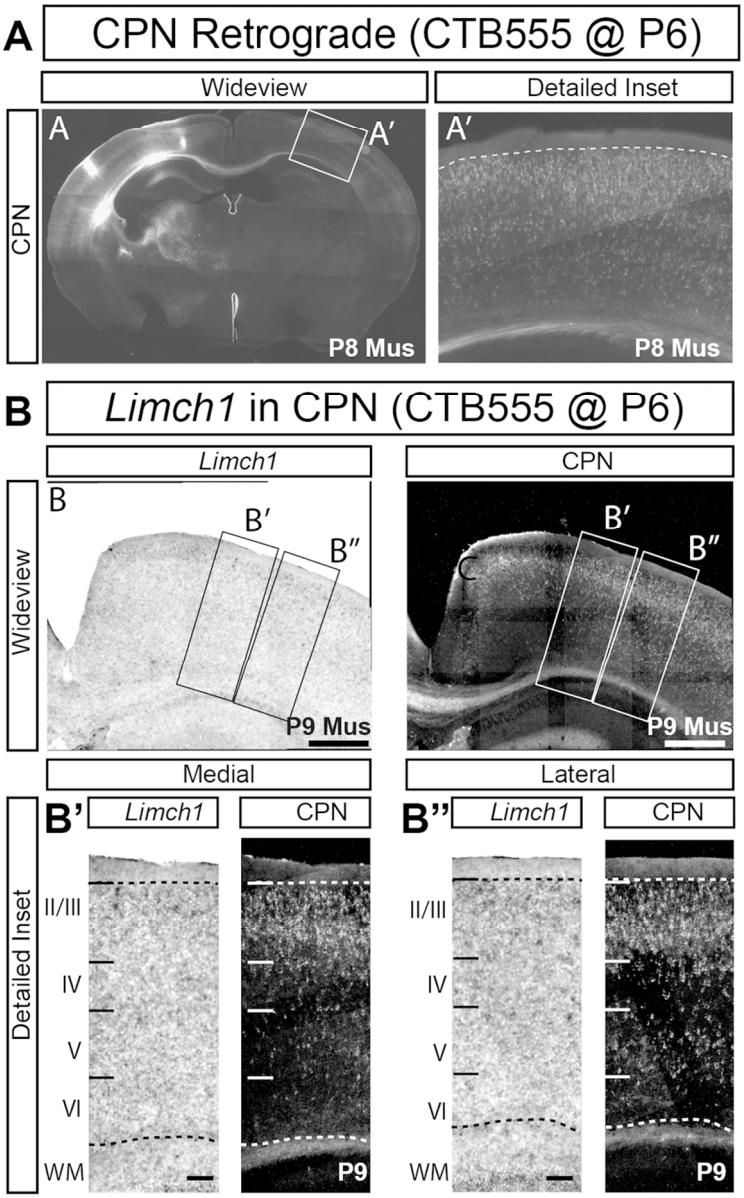

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(A) Retrograde labeling in mouse using CTB reveals CPN cell bodies in cortical layer II/III at P8, including superficial portions of layer II/III. (B) in situ hybridization combined with retrograde labeling using CTB reveals CPN cell bodies in superficial portions of layer II/III whose localization overlaps with expression of the CPN gene Limch1 in mouse, suggesting that the layer II expression of Limch1 in Macaque (Figure 4) might include CPN within that layer. (B') Detailed medial inset from (B). (B") Detailed lateral inset from (B).

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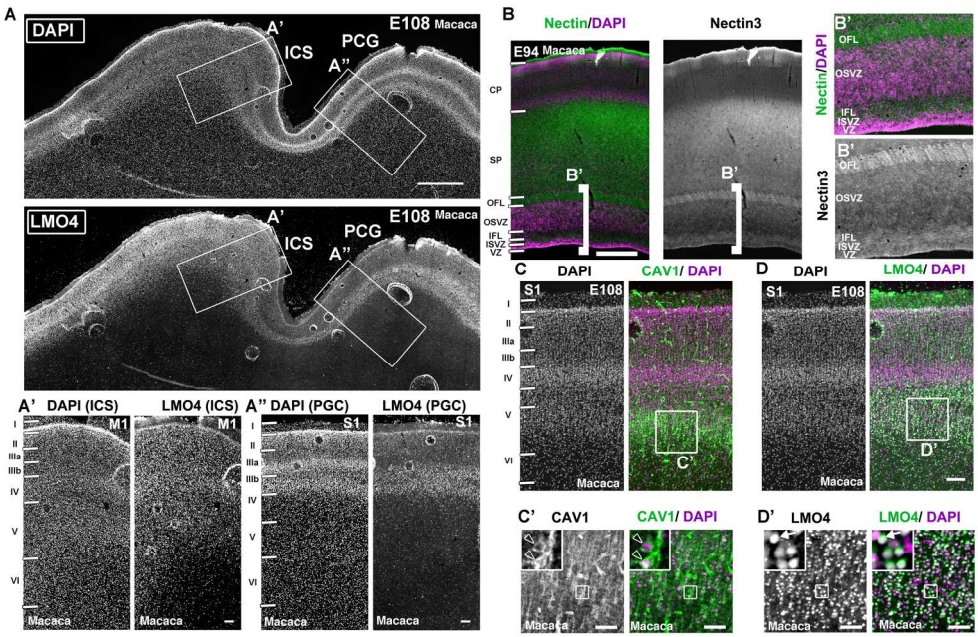

Figure 7: Functional areal and subcellular localization of CPN-expressed proteins suggests related functions in rodents and primates for conserved genes expressed by CPN populations.
(A, A', A") Areal restriction of LMO4 is conserved between mouse and macaque. LMO4 expression in motor cortex extends throughout all neocortical layers, and more restricted expression limited to deep layers is observed in somatosensory cortex.
(B, B', C, C', D. D') Three proteins with distinct subcellular localization in mouse CPN were selected for study of subcellular localization in macaque: 1) Nectin-3, with axonal white matter localization by superficial layer CPN axons in the CC; 2) CAV1, with cell body and neurite localization by a subpopulation of deep layer CPN; and 3) LMO4, with nuclear localization in deep layer CPN of somatosensory cortex. (B, B') At E94, Nectin-3 is localized specifically in white matter tracts in developing macaque cortex. There is low level localization in the cell-dense cortical plate, higher level localization throughout the cell-sparse subplate, and very high level, fiber-localization in both the outer and inner fibrous layers. (C, C') CAV1 is localized to neuronal cell membranes and neurites, as well as to developing blood vessels, as is seen in mouse ((Gaillard S et al. 2001; Boulware MI et al. 2007; Molyneaux BJ et al. 2009), and (D, D') LMO4 shows nuclear localization in deep layer somatosensory neocortical neurons, as has been shown in mouse. Empty arrowheads indicate membranous localization, and filled arrows indicate nuclear localization.

Scale bars: A, 1 mm; and A', A", 100 μm; B, C, D, 100 μm; C', D', 50 μm. E, embryonic day; P, postnatal day; ICS, incipient central sulcus; PCG, postcentral gyrus; S1, primary somatosensory area; M1, primary motor area; VZ, ventricular zone; SVZ, subventricular zone; SP, subplate; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; CP, cortical plate; roman numerals indicate cortical layers.

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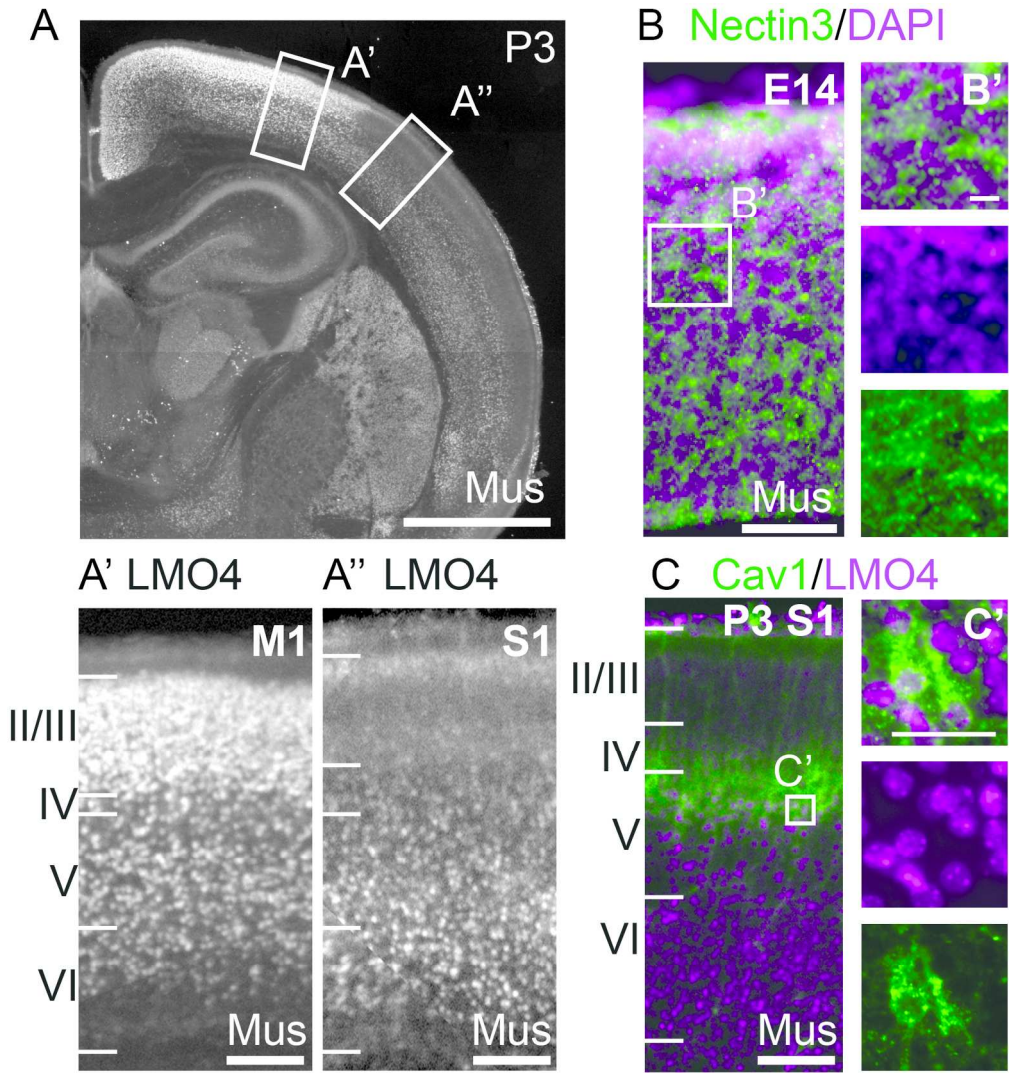

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Scale bars: A, 1 mm; and A', A", 100 μm; B, C, 100 μm; B', C', 25 μm. E, embryonic day; P, postnatal day; S1, primary somatosensory area; M1, primary motor area; roman numerals indicate cortical layers.

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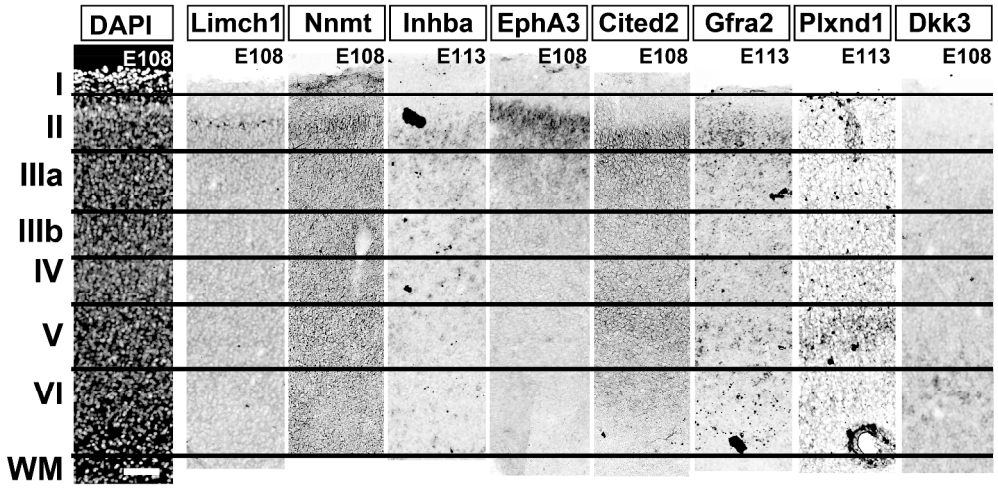

Figure S2: Sublaminar expression of CPN genes in E108 and 113 macaque reveals subpopulations defined by overlapping expression of multiple genes.
DAPI staining and in situ hybridization at E108 and E113 in macaque for representative genes in sagittal sections of cortex, shows molecularly distinct cortical populations. (E108: Limch1, Nnmt, EphA3, Cited2, Dkk3, N=3 technical replicates each); E113: Inhba, Gfra, Plxnd1, N=2 technical replicates each)
Solid lines identify layers. Scale bar: 100 μm. WM, white matter; roman numerals indicate cortical laminae.

400x196mm (300 x 300 DPI)

Gene	GenBank/ Ensembl ID	Left primer	Right primer	Amplicon
Chn2	NM_001261577.1	ATGAGCCTTTCACACCAA	ATGCTTTGCCTTCCATTTA	444 bp
Cited2	XM_001096152.2	AAAGAAATCAAACCCCCA	GCCACCAAAGGTAACAAT	900 bp
Dkk3	NM_001265749.1	CAGTTATCACGTCTGTGG	GAGCTGAATAAATGCACA	823 bp
Epha3	XM_001083136.1	GGCTGTGGAAGGTGTAGC	GGCTCTAAGCACCAGAGC	955 bp
Gfra2	XM_001105178.2	TTTGACATGACGCCCAAC	GGCATTTCTAGAGCCTCGT	757 bp
Inhba	XM_001098421.1	TTTCTGTTGGCAAGTTGCT	CATCCCCCTCCTCTTCTTT	781 bp
Limch	NW_001118139	GGCCAAGAAAGGAGTCA	CTCCATGAGAAGTCCTGG	517 bp
Nnmt	XM_001086727.1	GGAACAGAGTCAAGGGTC	CCACCAGGGAGAAAAGTC	414 bp
PlxnD	ENSMMUG000000127	GCAAGGTTCTCAACTCCA	CAGCTCTCAGGAATCTTCC	865 bp
Ptn	NM_001194354.1	TGCAACAAAGGCAGACTG	TCTCCTGTTTCTTGCCTTC	540 bp
Tmtc4	XM_001094711.2	ACCGGATGGCAATTAAAC	CAAGCTGCAAGGAGATTT	429 bp
Fezf2	(Arlotta et al., 2005)			

TableS1: Detailed Information About the Clones Used for *In Situ* Hybridization

Figure S1

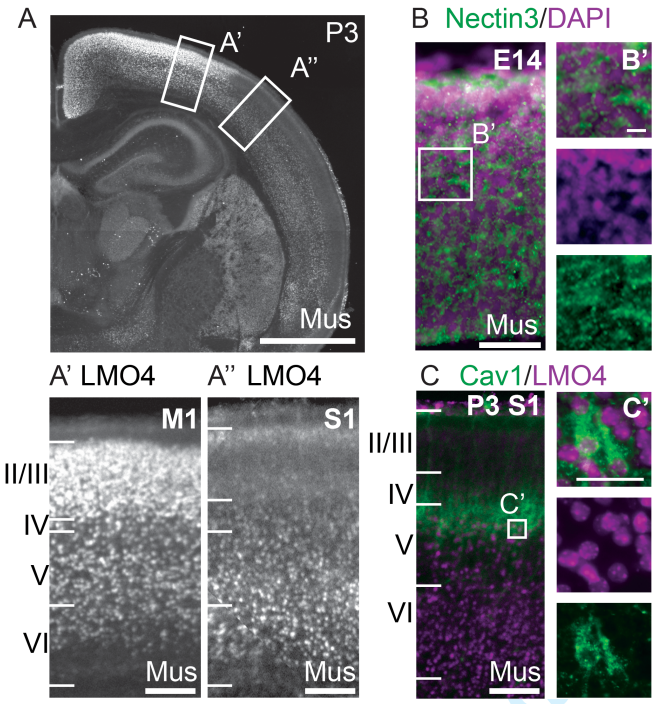

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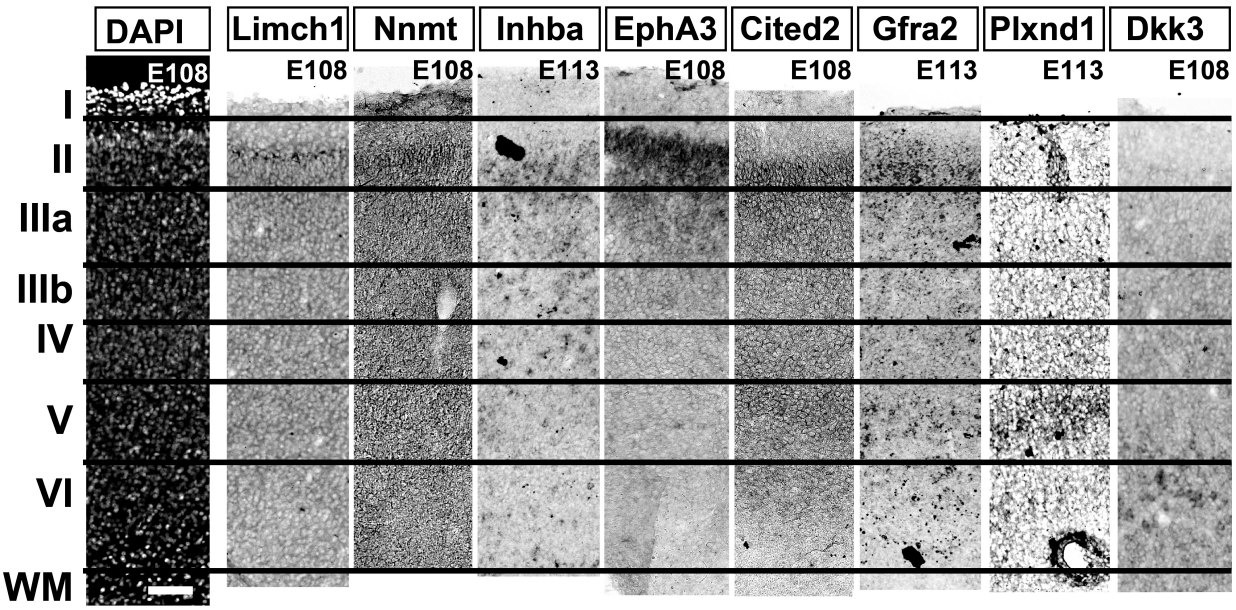
Figure S2



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Chn2	NM_001261577.1	ATGAGCCTTTCACACCAA	ATGCTTTGCCTTCCATTTA	444 bp
Cited2	XM_001096152.2	AAAGAAATCAAACCCCCA	GCCACCAAAGGTAACAAT	900 bp
Dkk3	NM_001265749.1	CAGTTATCACGTCTGTGG	GAGCTGAATAAATGCACA	823 bp
Epha3	XM_001083136.1	GGCTGTGGAAGGTGTAGC	GGCTCTAAGCACCAGAGC	955 bp
Gfra2	XM_001105178.2	TTTGACATGACGCCCAAC	GGCATTTCTAGAGCCTCGT	757 bp
Inhba	XM_001098421.1	TTTCTGTTGGCAAGTTGCT	CATCCCCCTCCTCTTCTTT	781 bp
Limch	NW_001118139	GGCCAAGAAAGGAGTCA	CTCCATGAGAAGTCCTGG	517 bp
Nnmt	XM_001086727.1	GGAACAGAGTCAAGGGTC	CCACCAGGGAGAAAAGTC	414 bp
PlxnD	ENSMMUG000000127	GCAAGGTTCTCAACTCCA	CAGCTCTCAGGAATCTTCC	865 bp
Ptn	NM_001194354.1	TGCAACAAAGGCAGACTG	TCTCCTGTTTCTTGCCTTC	540 bp
Tmtc4	XM_001094711.2	ACCGGATGGCAATTAAAC	CAAGCTGCAAGGAGATTT	429 bp
Fezf2	(Arlotta et al., 2005)			

TableS1: Detailed Information About the Clones Used for *In Situ* Hybridization